Role of Endothelin in Mediating Tumor Necrosis Factor-Induced Hypertension in Pregnant Rats

B. Babbette D. LaMarca, Kathy Cockrell, Elizabeth Sullivan, William Bennett, Joey P. Granger

Abstract—Hypertension during preeclampsia is associated with an increase in plasma levels of tumor necrosis factor (TNF)-α, a cytokine known to contribute to endothelial dysfunction. Recently, our laboratory reported that a 2-fold increase in plasma TNF-α produces hypertension in pregnant rats. Endothelin is also elevated in preeclampsia and endothelin synthesis is enhanced by TNF-α. The purpose of this study was to determine the role of endothelin in mediating TNF-α–induced hypertension in pregnant rats. To achieve this goal, TNF-α (50 ng/d for 5 days) was infused into control pregnant rats and pregnant rats treated with an endothelin receptor A antagonist, ABT 627 (5 mg/kg per day for 5 days). At day 19 of gestation, arterial pressure was measured and aorta, kidneys, and placentas were harvested. Infusion of TNF-α into pregnant rats increased plasma concentration of TNF-α (13.5 ± 0.8 to 28.0 ± 3.7 pg/mL) and arterial pressure (101 ± 2 to 122 ± 1 mm Hg). The increase in arterial pressure was associated with an increase in preproendothelin mRNA expression in placenta, aorta, and kidneys measured by real-time polymerase chain reaction (PCR). Pretreatment with the endothelin receptor A antagonist completely abolished the blood pressure response to TNF-α in pregnant rats (105 ± 1 versus 97 ± 2 mm Hg). In sharp contrast, the ET₄ receptor antagonist had no effect on arterial pressure in normal pregnant rats (97 ± 2 versus 101 ± 2 mm Hg). Moreover, chronic infusion of TNF-α had no significant effect on arterial pressure or renal preproendothelin levels in virgin rats. These results suggest an important role for endothelin in mediating TNF-α–induced hypertension in pregnant rats. (Hypertension. 2005;46:1-5.)

Key Words: blood pressure ● endothelin ● preeclampsia ● pregnancy

Preeclampsia is a multisystemic disorder of pregnancy that is estimated to affect 5% to 10% of all pregnancies in the United States. An important initiating event during preeclampsia is thought to be inadequate invasion of the trophoblast into the uterine spiral arteries leading to decreased placental perfusion. Subsequent release of circulating factors such as soluble vascular endothelial growth factor receptors and inflammatory cytokines including tumor necrosis factor (TNF)-α may serve as an important mediator of maternal endothelial activation and/or dysfunction. TNF-α mRNA and protein are expressed by the placenta during normal pregnancy; however, hypoxia results in a 2-fold increase in TNF production from placental explants. In women with preeclampsia, both placental TNF-α levels and plasma TNF levels are increased 2-fold. These changes in plasma TNF levels appear to be physiologically significant because our laboratory recently reported that a 2-fold increase in plasma TNF-α in results in significant increases in mean arterial pressure in pregnant rats but not virgin rats.

The physiological mechanisms whereby TNF-α increases in arterial pressure in pregnant rats are unclear. Several lines of evidence suggest that endothelin may be an important factor. Compelling evidence indicates that the endothelin system plays a role in regulating renal hemodynamics during normal pregnancy. Endothelin production is increased in women with preeclampsia. We have recently reported that the hypertension in response to chronic reductions in uteroplacental perfusion pressure in the pregnant rat is associated with significant increases in renal expression of preproendothelin and plasma TNF-α. Furthermore, selective blockade of the ET₄ receptor virtually abolished the hypertension in response to chronic reductions in uteroplacental perfusion pressure in the pregnant rat. Moreover, Marsden et al reported that TNF-α has a direct effect to increase ET-1 release and preproendothelin mRNA content in bovine renal artery and bovine glomerular capillary endothelial cells.

Although TNF-α has been reported to regulate endothelial expression of endothelin, the importance of endothelin in mediating the elevation in arterial pressure during TNF-α–induced hypertension in pregnant rats is unknown. Therefore, the purpose of this study was to determine the role of endothelin in mediating TNF-α–induced hypertension in pregnant rats. To achieve this goal, we examined the effects of TNF-α on endothelin production in virgin and pregnant rats.
rats and compared the blood pressure effects of TNF-α between control pregnant rats and pregnant rats treated with an endothelin type A receptor antagonist, ABT 627.

Methods

All studies were performed in virgin and timed pregnant Sprague-Dawley rats purchased from Harlan Inc (Indianapolis Ind). Animals were housed in a temperature-controlled room (23°C) with a 12:12 light:dark cycle. All experimental procedures executed in this study were in accordance with National Institutes of Health guidelines for use and care of animals. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi Medical Center.

Experimental Design

TNF-α (Biosource International, Camarillo Calif) was infused at a rate of 50 ng/d for 5 days (day 14 to 19 gestation) via mini-osmotic pumps (model 2002; Alzet Scientific Corporation, Palo Alto, Calif) in normal pregnant rats (n=16) and in pregnant rats (n=15) orally treated (drinking water) with the ETA receptor antagonist (ABT-627, 5 mg/kg per day for 5 days). Pregnant rats (n=16) receiving saline vehicle via mini-osmotic pumps and pregnant rats treated with ETα receptor antagonist alone served as controls. Rats were also surgically instrumented with a carotid catheter for subsequent arterial pressure measurement on day 19. At day 19 of gestation, arterial pressure was measured, blood samples were collected, kidneys, placentas and aortas were harvested, and litter size and pup weights were recorded. TNF-α was also infused at a rate of 50 ng/d for 5 days via mini-osmotic pumps into virgin rats (n=12), whereas untreated virgin rats (n=11) served as controls.

Measurement of Arterial Pressure in Chronically Instrumented Conscious Rats

Arterial pressure was determined in all groups of rats at day 19 of gestation. Pregnant rats were catheterized on day 18 of gestation under anesthesia using isolurane (Webster, Sterling, Mass) delivered by an anesthesia apparatus (Vaporizer for Forane Anesthetic, Ohio Medical Products, Madison, Wis). A catheter of V-3 tubing (SCI, Lake Hayasu City, Ariz) was inserted into the carotid artery for blood sampling and blood pressure monitoring. The catheter was tunneled to the back of the neck and exteriorized after implantation. On day 19 of gestation, pregnant rats were placed in individual restraining cages for arterial pressure measurements. Arterial pressure was monitored with a pressure transducer (Cobe III Transducer CDX Sema, Birmingham, Ala) and was recorded continuously for a 2-hour period after a 1-hour stabilization.

Determination of Serum TNF-α Levels

A rat TNF-α calorimetric sandwich enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn) was used for quantification of serum TNF-α levels between 12.5 and 800 pg/mL. This assay displayed a sensitivity level of 5 pg/mL and inter-assay variability of 10% and intra-assay of 5.1%.

Determination of Plasma Endothelin Levels

Endothelin levels were determined using the Parameter Human Endothelin enzyme-linked immunosorbent assay kit supplied by R&D Systems (Minneapolis, Minn). Endothelin was isolated from 1 mL of plasma in 1.5 mL of extraction solvent (40:1:5, acetone:1 N HCl:water). The extraction procedure was performed after the protocol outlined in the sample preparation of the manufacturer’s instructions. The assay displayed a sensitivity of 1.0 pg/mL, inter- and intra-assay variability of 10%.

Determination of Kidney and Placental Preproendothelin mRNA Levels

The cortex and medulla of the kidneys were separated immediately after harvesting and quickly frozen in liquid nitrogen (LN2) and stored at −80°C. Total RNA was extracted using the Totally RNA kit supplied by Ambion after the cortex and medulla were crushed in LN2 with a mortar and pestle. Isolation procedure was then performed as outlined in the instructions provided by the manufacturer.

Genomic DNA was digested with DNase1 following instructions outlined by Ambion. RNA was quantified spectrophotometrically using an Eppendorf BioPhotometer. cDNA was synthesized from 5 μg of RNA with Invitrogen’s Superscript II reverse-transcriptase using the following primers: preproendothelin forward 1: CTAGGTCTAAGCTGCTTGGC, and preproendothelin reverse 1: TCTTTGTCTGCTTGGC, supplied by custom primers from Life Technologies.

Real-time polymerase chain reaction (PCR) was performed using the BioRad Sylbe Green supernmix and iCycler using a nested forward primer; preproendothelin forward 2: CTAGGTCTAAGCTGCTTGGC, and the reverse primer outlined. Invitrogen’s reverse-transcription PCR primer control kit was used to amplify β-actin transcripts as control. Levels of mRNA expression was calculated using the mathematical formulas for delta/delta CT recommended by Applied Biosystems (Applied Biosystems User Bulletin, No. 2, 1997). Statistical analysis of real-time PCR results was performed using the mean normalized cycle threshold (delta/delta CT) values and standard deviations analyzed by 1-way ANOVA and Tukey–Kramer multiple comparison test.

Determination of Preproendothelin Levels From Aorta

The aortas were quick-frozen in LN2 and stored at −80°C. Total RNA was extracted using the Totally RNA kit supplied by Ambion after the aortas were crushed in LN2 with a mortar and pestle. Crushed tissue was suspended in 500 μL denaturation solution and the remaining isolation procedure was then performed as outlined in the instructions provided by the manufacturer. After air-drying, the isolated RNA was suspended in 25 μL of DEPC water, genomic DNA was digested and real-time PCR was performed as outlined.

Results

Arterial Pressure Responses to TNF-α in Normal Pregnant Rats and ETα Receptor Antagonist-Treated Pregnant Rats

Chronic infusion of 50 ng/d of TNF-α in pregnant rats resulted in significant increases in arterial pressure relative to control rats. Mean arterial pressure (Figure 1) averaged $122\pm1$ mm Hg in the TNF-α-treated pregnant rats at day 19 of pregnancy. This was a significant increase as compared with an average of 101±2 mm Hg in control pregnant rats. In contrast, mean arterial pressure of rats treated with TNF-α and ETα receptor antagonist averaged 97±2 mm Hg, whereas...
pregnant rats treated with ETα receptor antagonist alone had arterial pressures of 105±1 mm Hg.

There was no difference in pup weight (2.4±0.05 grams versus 2.38±0.02 grams versus 2.4±0.02 grams versus 2.34±0.02 grams) or litter size (14±0.19 grams versus 14±0.09 grams versus 13±0.13 grams versus 14±0.15 grams) between normal pregnant, TNF-α–treated, ETA receptor antagonist-treated, or TNF-α plus ETα receptor antagonist pregnant rats, respectively.

**TNF-α Levels in Control and TNF-α–Treated Pregnant Rats**

A significant elevation in serum TNF-α levels was achieved in TNF-α–treated rats as compared with normal pregnant rats (28.0±3.7 versus 13.5±0.8 pg/mL). TNF-α was increased in TNF-α/ETA receptor antagonist pregnant versus ETα receptor antagonist pregnant rats as well (23.0±2.8 versus 11.7±3.0 pg/mL) (Figure 2).

**Plasma Endothelin Levels in Control and TNF-α–Treated Pregnant Rats**

There were no significant differences in plasma endothelin levels between control pregnant and TNF-α–treated pregnant rats (1.7±0.3 versus 2.2±0.3 pg/mL, respectively). However, as expected, a significant increase in plasma endothelin was noted in ETα receptor antagonist-treated pregnant rats as well as TNF-α–treated and ETα receptor antagonist-treated pregnant rats (3.6±0.6 versus 3.4±0.8 pg/mL, respectively). However, incomplete cross-reactivity between the human antibody to rat endothelin may account for the inability to sufficiently detect changes in plasma levels of the protein.

**Preproendothelin mRNA Levels in Aorta, Placenta, and Kidneys in Control and TNF-α–Treated Pregnant Rats**

Real-time PCR was used to measure preproendothelin in the placenta, aorta, and the renal cortex and the medulla. Preproendothelin mRNA levels in the renal medulla and placenta of the TNF-α–treated rats were increased by ~5-fold as compared with normal pregnant rats. Preproendothelin mRNA levels in the aorta were increased by 100% in pregnant rats treated with TNF-α compared with levels measured in normal pregnant rats (Figure 3).

**Arterial Pressure and Endothelin Responses to TNF-α in Virgin Rats**

Whereas chronic infusion of 50 ng/d of TNF-α in virgin rats resulted in an increase in plasma levels of TNF by 20 pg/mL, there was no significant increases in arterial pressure relative to control rats (Figure 4). Mean arterial pressure averaged 125±3 mm Hg in the TNF-α–treated virgin rats compared with 125±2 mm Hg in virgin control rats. There were also no significant differences in plasma endothelin levels (2.8±0.3 versus 2.2±0.3 pg/mL) or renal preproendothelin levels (6.3±0.7 versus 7.7±0.5) between the virgin controls and TNF-treated virgin rats.

**Discussion**

Although reductions in blood flow to the uteroplacental unit are known to result in cardiovascular and renal abnormalities consistent with the pathophysiological features of human pregnancy-induced hypertension, the physiological mechanisms linking placental ischemia with the abnormalities in the maternal circulation are unclear.3,4,5,7,21–24 Several lines of evidence support the hypothesis that the ischemic placenta contributes to endothelial cell activation/dysfunction of the
maternal circulation by enhancing the synthesis of cytokines such as TNF-α. TNF-α is an inflammatory cytokine that has been shown to induce structural as well as functional alterations in endothelial cells.25 TNF-α also enhances the formation of a number of endothelial cell substances such as endothelin and reduces acetylcholine-induced vasodilatation.25 Also supporting a potential role of TNF-α in preeclampsia are findings that plasma levels of TNF-α are significantly elevated in women with preeclampsia by ~2-fold.25 We have also recently reported that chronic infusion of TNF-α in pregnant rats, at a rate to mimic plasma levels (2- to 3-fold increase) observed in women with preeclampsia, resulted in significant increases in blood pressure. The data obtained in the present study not only confirm our previous findings that chronic infusion TNF-α into pregnant rats increases arterial pressure by ~20 mm Hg but also extend these findings by demonstrating an important role for endothelin in mediating TNF-induced hypertension in pregnant rats.

Compelling evidence support an important role for endothelin in preeclampsia. Plasma concentration of endothelin has been measured in a number of studies involving normal pregnant women and women with preeclampsia.14–16 Some, but not all, studies have found higher endothelin plasma concentrations of ~2- to 3-fold in women with preeclampsia.14 Typically, plasma levels of endothelin are highest during the latter stage of the disease, suggesting that endothelin may not be involved in the initiation of preeclampsia, but rather in the progression of disease into a malignant phase.14–17

Although some studies have reported no significant changes in circulating levels of endothelin during pregnancy-induced hypertension, a role for endothelin as a paracrine or autocrine agent in preeclampsia has been suggested.25,26 A number of studies have found significant increases in tissue mRNA expression of preproendothelin in humans with pre-eclampsia.26 We have recently reported that the hypertension in response to chronic reductions in utero-placental perfusion pressure in the pregnant rat is associated with significant increases in renal expression of preproendothelin and serum levels of TNF-α.18,19 Furthermore, selective blockade of the ETA receptor virtually abolished the hypertension in response to chronic reductions in utero-placental perfusion pressure in the pregnant rat.18

Although TNF-α has been reported to regulate endothelial expression of endothelin, the importance of endothelin in mediating the elevation in arterial pressure during TNF-α-induced hypertension in pregnant rats is unknown. To address this important unanswered question, we examined the effects of TNF-α on endothelin production in pregnant rats. We found no significant differences in plasma levels of endothelin between the normal pregnant rats and pregnant rats infused with TNF-α. In contrast, we found preproendothelin mRNA levels to be elevated in the kidney, placenta, and aorta in pregnant rats treated with TNF-α. Preproendothelin mRNA levels in the kidney and placenta were increased by ~5-fold in the TNF-α-infused pregnant rats. Preproendothelin mRNA levels in the aorta of TNF-α-infused pregnant rats were elevated by >2-fold. These findings are comparable to women with mild preeclampsia and the RUPP model of preeclampsia in which plasma levels of endothelin are normal while tissue levels of preproendothelin mRNA are elevated. Therefore, the increase in arterial pressure produced by a 2- to 3-fold elevation in plasma levels of TNF-α in pregnant rats is associated with significant increases in local production of endothelin in the kidney, placenta, and vasculature. To determine the role of endothelin in mediating TNF-α-induced hypertension in pregnant rats, we compared the blood pressure responses to a 5-day (days 14 to 19 of gestation) infusion of TNF-α in control pregnant rats and pregnant rats treated with an endothelin receptor A antagonist, ABT 627. Infusion of TNF-α in control pregnant rats increased arterial pressure by >20 mm Hg. This increase is comparable to what we previously reported in pregnant rats infused with TNF at a rate to mimic levels observed in women with preeclampsia. In sharp contrast to the finding in normal pregnant rats, we found that the increase in mean arterial pressure in response to TNF-α was completely abolished in pregnant rats treated with the endothelin receptor A antagonist. We also found that the endothelin type A receptor antagonist had no effect on mean arterial pressure in normal pregnant rats. Moreover, chronic infusion of TNF-α had no significant effect on arterial pressure or renal preproendothelin levels in virgin rats. Collectively, these findings suggest that endothelin, via ETA receptor activation, plays an important role in mediating TNF-α–induced hypertension in pregnant rats.
Compelling evidence indicates that the endothelin system, via activation of endothelin type B receptors, plays a role in mediating the renal vasodilation during normal pregnancy. Thus, another potential mechanism whereby TNF-α may influence renal hemodynamics and arterial pressure regulation during pregnancy is by altering the distribution of endothelin type A and B receptors within the kidney. Because this possibility was not explored in this study, the importance of TNF-α in altering endothelin type A and B receptors distribution during pregnancy remains unclear.

**Perspectives**

Preeclampsia, which affects 5% to 10% of all pregnancies in the United States, is a multisystemic disorder of pregnancy that is associated with hypertension and endothelial dysfunction. Despite being one of the leading causes of maternal and perinatal morbidity and mortality, the pathophysiological mechanisms underlying the hypertension during preeclampsia are unknown. Increases in circulating factors such as soluble vascular endothelial growth factor receptors1 and inflammatory cytokines2,3 may serve as important mediators of maternal endothelial activation and/or dysfunction in preeclampsia. In this study, we report that the increase in arterial pressure produced by a 2- to 3-fold elevation in plasma levels of TNF-α in pregnant rats is associated with significant increases in local production of endothelin in the kidney, placenta, and vasculature. Moreover, we found that the increase in mean arterial pressure in response to TNF was completely abolished in pregnant rats treated with the endothelin type A receptor antagonist. These findings suggest that endothelin, via ETA receptor activation, plays an important role in mediating TNF-α-induced hypertension in pregnant rats. Whether endothelin plays a role in mediating the hypertension induced by soluble vascular endothelial growth factor receptors in pregnant rats is unknown but remains to be an important area of investigation.

Though we have shown that the endothelin system plays an important role in mediating the hypertension in response to reductions in uterine perfusion pressure and in response to chronic elevations in serum levels of TNF-α in pregnant rats, the usefulness of selective endothelin type A receptor antagonists for the treatment of hypertension in women with preeclampsia remains unclear. This important question will not be answered until well-controlled clinical studies, using specific inhibitors of the endothelin system, are performed in women with preeclampsia.

**Acknowledgments**

This work was supported by National Institutes of Health grants HL38499 and HL51971.

**References**

19. LaMarca BBD, Roberts L, Cockrell K, Bain JC, Granger JP. Hypertension produced by chronic reductions in uterine perfusion pressure in the pregnant rat is associated with enhanced production of TNF alpha and IL-6. FASEB. 2005;19:A1598.
Role of Endothelin in Mediating Tumor Necrosis Factor-Induced Hypertension in Pregnant Rats

B. Babbette D. LaMarca, Kathy Cockrell, Elizabeth Sullivan, William Bennett and Joey P. Granger

Hypertension. published online May 31, 2005;

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2005/05/31/01.HYP.0000169152.59854.36.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/